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(21) International Application Number: PCT/US98/04278 (22) International Filing Date: 5 March 1998 (05.03.98) (30) Priority Data: 08/812,871 6 March 1997 (06.03.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/812,871 (CIP) Filed on 6 March 1997 (06.03.97) (71) Applicant (for all designated States except US): INCYTE PHARMACEUTICALS, INC. [US/US]; 3174 Porter Drive, Palo Alto, CA 94304 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): AU-YOUNG, Janice [US/US]; 1419 Kains Avenue, Berkeley, CA 94702 (US). GUEGLER, Karl, J. [CH/US]; 1048 Oakland Avenue, Menlo Park, CA 94025 (US). CHENG, Muzong [US/US]; 648 Alcatraz Avenue #301, Oakland, CA 94609 (US). (74) Agent: BILLINGS, Lucy, J.; Incyte Pharmaceuticals, Inc., 3174 Porter Drive, Palo Alto, CA 94304 (US).			(81) Designated States: AT, AU, BR, CA, CH, CN, DE, DK, ES, FI, GB, IL, JP, KR, MX, NO, NZ, RU, SE, SG, US, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: HUMAN CHEMOKINE RECEPTOR-LIKE CHEMOKINE			
(57) Abstract  The present invention provides a human chemokine receptor-like protein (NHCR) and polynucleotides which identify and encode NHCR. The invention also provides expression vectors, host cells, agonists, antibodies, and antagonists. The invention also provides methods for treating disorders associated with expression of NHCR.			

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## HUMAN CHEMOKINE RECEPTOR-LIKE CHEMOKINE

## TECHNICAL FIELD

This invention relates to nucleic acid and amino acid sequences of a novel human chemokine receptor-like protein and to the use of these sequences in the diagnosis, prevention, and treatment of disorders associated with inflammation and viral infection.

## BACKGROUND ART

Chemokines are a large family of low molecular weight, inducible, secreted, proinflammatory cytokines which are produced by various cell types. They have been divided into several subfamilies on the basis of the positions of their conserved cysteines. The CXC family includes interleukin-8 (IL-8), growth regulatory gene, neutrophil-activating peptide-2, and platelet factor 4 (PF-4). Although IL-8 and PF-4 are both polymorphonuclear chemoattractants, angiogenesis is stimulated by IL-8 and inhibited by PF-4. The CC family includes monocyte chemoattractant protein-1 (MCP-1), RANTES (regulated on activation, normal T cell-expressed and secreted), macrophage inflammatory proteins (MIP-1 $\alpha$ , MIP-1 $\beta$ ), and eotaxin. MCP-1 is secreted by numerous cell types including endothelial, epithelial, and hematopoietic cells, and is a chemoattractant for monocytes and CD45RO+ lymphocytes (Proost, P. (1996) *Int J. Clin. Lab. Res.* 26:211-223; Raport, C. J. (1996) *J. Biol. Chem.* 271:17161-17166).

Cells respond to chemokines through G-protein-coupled receptors. These receptors are seven transmembrane molecules which transduce their signal through heterotrimeric GTP-binding proteins. Stimulation of the GTP-binding protein complex by activated receptor leads to the exchange of guanosine diphosphate for guanosine triphosphate and regulates the activity of effector molecules. There are distinct classes of each of the subunits which differ in activity and specificity and can elicit inhibitory or stimulatory responses. When stimulation of the known cytokine receptors shows agonist-dependent inhibition of adenylyl cyclase and mobilization of intracellular calcium, the receptor coupling to G $\alpha_i$  subunits (Myers, S. J. et al (1995) *J. Biol. Chem.* 270:5786-5792).

Chemokine receptors play a major role in the mobilization and activation of cells of the immune system. The effects of receptor stimulation are dependent on the cell type and include chemotaxis, proliferation, differentiation, and production of cytokines. Chemokine stimulation produces changes in vascular endothelium, chemotaxis to sites of inflammation, and activates the effector functions of cells (Taub, D. D. (1996) *Cytokine Growth Factor Rev.* 7:355-376).

The chemokine receptors display a range of sequence diversity and ligand promiscuity. The known chemokine receptor protein sequence identities range from 22 to 40%, and certain

receptors can respond to multiple ligands. Although mainly expressed in immune cells, viral homologues are expressed by human cytomegalovirus and Herpesvirus saimiri. The chemokine receptor known as the Duffy blood group antigen binds both CC and CXC family chemokines and serves as the receptor on erythrocytes for the malarial parasite Plasmodium vivax. Members  
5 of the chemokine receptor family are used as co-receptors with CD4 for HIV-1 entry into target cells. Several receptors have recently been cloned. The human chemokine receptor, R12, was isolated by cross-hybridization of an APJ/R20 probe on a human genomic library. R12 is most similar to the R20 orphan receptor (which has  
homology with the angiotensin receptor) and shows between 22 and 26% homology to  
10 characterized chemokine receptors including IL-8A and B, and MCP-1 $\alpha$  and 1 $\beta$ . (Murphy, P.M. (1994) Annu. Rev. Immunol. 12:593-633; Raport, C.J. et al (1996) J. Leuk. Biol. 59:18-23; He, J. et al (1997) Nature 385:645-649).

The discovery of proteins related to human chemokine receptor R12 and the polynucleotides encoding them satisfies a need in the art by providing new compositions useful in  
15 diagnosis and treatment of disorders associated with inflammation and viral infection.

#### DISCLOSURE OF THE INVENTION

The present invention features a novel human chemokine receptor-like protein hereinafter designated NHCR and characterized as having similarity to human chemokine receptor R12.

Accordingly, the invention features a substantially purified NHCR having the amino acid  
20 sequence shown in SEQ ID NO:1.

One aspect of the invention features isolated and substantially purified polynucleotides that encode NHCR. In a particular aspect, the polynucleotide is the nucleotide sequence of SEQ ID NO:2.

The invention also relates to a polynucleotide sequence comprising the complement of  
25 SEQ ID NO:2 or variants thereof. In addition, the invention features polynucleotide sequences which hybridize under stringent conditions to SEQ ID NO:2.

The invention additionally features nucleic acid sequences encoding polypeptides, oligonucleotides, peptide nucleic acids (PNA), fragments, portions or antisense molecules thereof, and expression vectors and host cells comprising polynucleotides that encode NHCR.  
30 The present invention also features antibodies which bind specifically to NHCR, and pharmaceutical compositions comprising substantially purified NHCR. The invention also features the use of agonists and antagonists of NHCR. The invention also features a method for treating disorders associated with decreased NHCR by administering NHCR and a method for

treating disorders associated with increased NHCR by administering an antagonist to NHCR.

### BRIEF DESCRIPTION OF DRAWINGS

Figures 1A, 1B, 1C, 1D and 1E show the amino acid sequence (SEQ ID NO:1) and nucleic acid sequence (SEQ ID NO:2) of NHCR. The alignment was produced using  
5 MacDNASIS PRO™ software (Hitachi Software Engineering Co., Ltd., San Bruno, CA).

Figures 2A and 2B shows the amino acid sequence alignment between NHCR (SEQ ID NO:1) and a human chemokine receptor (GI 992700; SEQ ID NO:3). The alignment was produced using the multisequence alignment program of DNASTAR™ software (DNASTAR Inc, Madison WI).

10 Figures 3A and 3B show the hydrophobicity plots (MacDNASIS PRO software) for NHCR and human chemokine receptor, respectively; the positive X axis reflects amino acid position, and the negative Y axis, hydrophobicity.

### MODES FOR CARRYING OUT THE INVENTION

Before the present proteins, nucleotide sequences, and methods are described, it is  
15 understood that this invention is not limited to the particular methodology, protocols, cell lines, vectors, and reagents described as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms “a”,  
20 “an”, and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to “a host cell” includes a plurality of such host cells, reference to the “antibody” is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same  
25 meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the cell lines, vectors, and methodologies  
30 which are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

**DEFINITIONS**

"Nucleic acid sequence" as used herein refers to an oligonucleotide, nucleotide, or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand.

- 5 Similarly, "amino acid sequence" as used herein refers to an oligopeptide, peptide, polypeptide, or protein sequence, and fragments or portions thereof, and to naturally occurring or synthetic molecules.

- Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms, such as
- 10 "polypeptide" or "protein" are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

- "Peptide nucleic acid", as used herein, refers to a molecule which comprises an oligomer to which an amino acid residue, such as lysine, and an amino group have been added. These small molecules, also designated anti-gene agents, stop transcript elongation by binding to their
- 15 complementary strand of nucleic acid (Nielsen, P.E. et al. (1993) Anticancer Drug Des. 8:53-63).

NHCR, as used herein, refers to the amino acid sequences of substantially purified NHCR obtained from any species, particularly mammalian, including bovine, ovine, porcine, murine, equine, and preferably human, from any source whether natural, synthetic, semi-synthetic, or recombinant.

- 20 "Consensus", as used herein, refers to a nucleic acid sequence which has been resequenced to resolve uncalled bases, or which has been extended using XL-PCR™ (Perkin Elmer, Norwalk, CT) in the 5' and/or the 3' direction and resequenced, or which has been assembled from the overlapping sequences of more than one Incyte clone using the GELVIEW™ Fragment Assembly system (GCG, Madison, WI), or which has been both extended and
- 25 assembled.

- A "variant" of NHCR, as used herein, refers to an amino acid sequence that is altered by one or more amino acids. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, e.g., replacement of a
- 30 glycine with a tryptophan. Similar minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNASTAR software.

A "deletion", as used herein, refers to a change in either amino acid or nucleotide sequence in which one or more amino acid or nucleotide residues, respectively, are absent.

An "insertion" or "addition", as used herein, refers to a change in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid or nucleotide residues, respectively, as compared to the naturally occurring molecule.

A "substitution", as used herein, refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

The term "biologically active", as used herein, refers to a protein having structural, regulatory, or biochemical functions of a naturally occurring molecule. Likewise, "immunologically active" refers to the capability of the natural, recombinant, or synthetic NHCR, or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

The term "agonist", as used herein, refers to a molecule which, when bound to NHCR, causes a change in NHCR which modulates the activity of NHCR. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules which bind to NHCR.

The terms "antagonist" or "inhibitor", as used herein, refer to a molecule which, when bound to NHCR, blocks or modulates the biological or immunological activity of NHCR. Antagonists and inhibitors may include proteins, nucleic acids, carbohydrates, or any other molecules which bind to NHCR.

The term "modulate", as used herein, refers to a change or an alteration in the biological activity of NHCR. Modulation may be an increase or a decrease in protein activity, a change in binding characteristics, or any other change in the biological, functional or immunological properties of NHCR.

The term "mimetic", as used herein, refers to a molecule, the structure of which is developed from knowledge of the structure of NHCR or portions thereof and, as such, is able to effect some or all of the actions of NHCR-like molecules.

The term "derivative", as used herein, refers to the chemical modification of a nucleic acid encoding NHCR or the encoded NHCR. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group. A nucleic acid derivative would encode a polypeptide which retains essential biological characteristics of the natural molecule.

The term "substantially purified", as used herein, refers to nucleic or amino acid sequences that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and most preferably 90% free from other components with which

they are naturally associated.

"Amplification" as used herein refers to the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction (PCR) technologies well known in the art (Dieffenbach, C.W. and G.S. Dveksler (1995) PCR Primer, a Laboratory  
5 Manual, Cold Spring Harbor Press, Plainview, NY).

The term "hybridization", as used herein, refers to any process by which a strand of nucleic acid binds with a complementary strand through base pairing.

The term "hybridization complex", as used herein, refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen binds between complementary  
10 G and C bases and between complementary A and T bases; these hydrogen bonds may be further stabilized by base stacking interactions. The two complementary nucleic acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution (e.g.,  $C_0t$  or  $R_0t$  analysis) or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid support (e.g., membranes, filters, chips,  
15 pins or glass slides to which cells have been fixed for in situ hybridization).

The terms "complementary" or "complementarity", as used herein, refer to the natural binding of polynucleotides under permissive salt and temperature conditions by base-pairing. For example, for the sequence "A-G-T" binds to the complementary sequence "T-C-A".

Complementarity between two single-stranded molecules may be "partial", in which only some  
20 of the nucleic acids bind, or it may be complete when total complementarity exists between the single stranded molecules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, which depend upon binding between nucleic acids strands.

25 The term "homology", as used herein, refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity). A partially complementary sequence is one that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid; it is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined  
30 using a hybridization assay (Southern or northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (i.e., the hybridization) of a completely homologous sequence or probe to the target sequence under conditions of low stringency. This is not to say that conditions of low



stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (i.e., selective) interaction. The absence of non-specific binding may be tested by the use of a second target sequence which lacks even a partial degree of complementarity (e.g., less than about 30% identity); in the absence of  
5 non-specific binding, the probe will not hybridize to the second non-complementary target sequence.

As known in the art, numerous equivalent conditions may be employed to comprise either low or high stringency conditions. Factors such as the length and nature (DNA, RNA, base composition) of the sequence, nature of the target (DNA, RNA, base composition, presence in  
10 solution or immobilization, etc.), and the concentration of the salts and other components (e.g., the presence or absence of formamide, dextran sulfate and/or polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of either low or high stringency different from, but equivalent to, the above listed conditions.

The term "stringent conditions", as used herein, is the "stringency" which occurs within a  
15 range from about  $T_m - 5^\circ\text{C}$  ( $5^\circ\text{C}$  below the melting temperature ( $T_m$ ) of the probe) to about  $20^\circ\text{C}$  to  $25^\circ\text{C}$  below  $T_m$ . As will be understood by those of skill in the art, the stringency of hybridization may be altered in order to identify or detect identical or related polynucleotide sequences.

The term "antisense", as used herein, refers to nucleotide sequences which are  
20 complementary to a specific DNA or RNA sequence. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. Antisense molecules may be produced by any method, including synthesis by ligating the gene(s) of interest in a reverse orientation to a viral promoter which permits the synthesis of a complementary strand. Once introduced into a cell, this transcribed strand combines with natural sequences  
25 produced by the cell to form duplexes. These duplexes then block either the further transcription or translation. In this manner, mutant phenotypes may be generated. The designation "negative" is sometimes used in reference to the antisense strand, and "positive" is sometimes used in reference to the sense strand.

The term "portion", as used herein, with regard to a protein (as in "a portion of a given  
30 protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid. Thus, a protein "comprising at least a portion of the amino acid sequence of SEQ ID NO:1" encompasses the full-length human NHCR and fragments thereof.

"Transformation", as defined herein, describes a process by which exogenous DNA enters and changes a recipient cell. It may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method is  
5 selected based on the host cell being transformed and may include, but is not limited to, viral infection, electroporation, lipofection, and particle bombardment. Such "transformed" cells include stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome. They also include cells which transiently express the inserted DNA or RNA for limited periods of time.

10 The term "antigenic determinant", as used herein, refers to that portion of a molecule that makes contact with a particular antibody (i.e., an epitope). When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as antigenic determinants. An antigenic  
15 determinant may compete with the intact antigen (i.e., the immunogen used to elicit the immune response) for binding to an antibody.

The terms "specific binding" or "specifically binding", as used herein, in reference to the interaction of an antibody and a protein or peptide, mean that the interaction is dependent upon the presence of a particular structure (i.e., the antigenic determinant or epitope) on the protein; in  
20 other words, the antibody is recognizing and binding to a specific protein structure rather than to proteins in general. For example, if an antibody is specific for epitope "A", the presence of a protein containing epitope A (or free, unlabeled A) in a reaction containing labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody.

The term "sample", as used herein, is used in its broadest sense. A biological sample  
25 suspected of containing nucleic acid encoding NHCR or fragments thereof may comprise a cell, chromosomes isolated from a cell (e.g., a spread of metaphase chromosomes), genomic DNA (in solution or bound to a solid support such as for Southern analysis), RNA (in solution or bound to a solid support such as for northern analysis), cDNA (in solution or bound to a solid support), an extract from cells or a tissue, and the like.

30 The term "correlates with expression of a polynucleotide", as used herein, indicates that the detection of the presence of ribonucleic acid that is similar to SEQ ID NO:2 by northern analysis is indicative of the presence of mRNA encoding NHCR in a sample and thereby correlates with expression of the transcript from the polynucleotide encoding the protein.

"Alterations" in the polynucleotide of SEQ ID NO: 2, as used herein, comprise any alteration in the sequence of polynucleotides encoding NHCR including deletions, insertions, and point mutations that may be detected using hybridization assays. Included within this definition is the detection of alterations to the genomic DNA sequence which encodes NHCR (e.g., by alterations in the pattern of restriction fragment length polymorphisms capable of hybridizing to SEQ ID NO:2), the inability of a selected fragment of SEQ ID NO: 2 to hybridize to a sample of genomic DNA (e.g., using allele-specific oligonucleotide probes), and improper or unexpected hybridization, such as hybridization to a locus other than the normal chromosomal locus for the polynucleotide sequence encoding NHCR (e.g., using fluorescent in situ hybridization [FISH] to metaphase chromosomes spreads).

As used herein, the term "antibody" refers to intact molecules as well as fragments thereof, such as Fa, F(ab')<sub>2</sub>, and Fv, which are capable of binding the epitopic determinant. Antibodies that bind NHCR polypeptides can be prepared using intact polypeptides or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or peptide used to immunize an animal can be derived from the transition of RNA or synthesized chemically, and can be conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin and thyroglobulin. The coupled peptide is then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

The term "humanized antibody", as used herein, refers to antibody molecules in which amino acids have been replaced in the non-antigen binding regions in order to more closely resemble a human antibody, while still retaining the original binding ability.

#### THE INVENTION

The invention is based on the discovery of a novel human chemokine receptor-like protein, (NHCR), the polynucleotides encoding NHCR, and the use of these compositions for the diagnosis, prevention, or treatment of disorders associated with inflammation and infection.

Nucleic acids encoding the human NHCR of the present invention were first identified in Incyte Clone 568987 from the macrophage cDNA library MMLR3DT01 through a computer-generated search for amino acid sequence alignments. The complete nucleotide sequence, SEQ ID NO:2, was derived from extension of Incyte clone 56897.

In one embodiment, the invention encompasses a polypeptide comprising the amino acid sequence of SEQ ID NO:1, as shown in Fig. 1. NHCR is 333 amino acids in length and has chemical and structural homology with human chemokine receptor (SEQ ID NO:3). In particular, NHCR and human chemokine receptor share 26% identity. Both NHCR and human

chemokine receptor contain a G-protein receptor motif, from V<sub>108</sub> - I<sub>124</sub> and A<sub>117</sub> - I<sub>133</sub>, respectively. In addition, NHCR and human chemokine receptor have potential amino terminal Asn-glycosylation sites, at N<sub>10</sub> and at N<sub>14</sub>, respectively, and a carboxy-terminal amidation site, at M<sub>310</sub> and at L<sub>312</sub>, respectively. As illustrated by Figs. 3A and 3B, NHCR and human cytokine  
5 receptor have rather similar hydrophobicity plots.

The invention also encompasses NHCR variants. A preferred NHCR variant is one having at least 80%, and more preferably 90%, amino acid sequence identity to the NHCR amino acid sequence (SEQ ID NO:1). A most preferred NHCR variant is one having at least 95% amino acid sequence identity to SEQ ID NO:1.

10 The invention also encompasses polynucleotides which encode NHCR. Accordingly, any nucleic acid sequence which encodes the amino acid sequence of NHCR can be used to generate recombinant molecules which express NHCR. In a particular embodiment, the invention encompasses the polynucleotide comprising the nucleic acid sequence of SEQ ID NO:2 as shown in Figures 1A, 1B, 1C, 1D and 1E.

15 It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding NHCR, some bearing minimal homology to the nucleotide sequences of any known and naturally occurring gene, may be produced. Thus, the invention contemplates each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices. These  
20 combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring NHCR, and all such variations are to be considered as being specifically disclosed.

Although nucleotide sequences which encode NHCR and its variants are preferably capable of hybridizing to the nucleotide sequence of the naturally occurring NHCR under  
25 appropriately selected conditions of stringency, it may be advantageous to produce nucleotide sequences encoding NHCR or its derivatives possessing a substantially different codon usage. Codons may be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence  
30 encoding NHCR and its derivatives without altering the encoded amino acid sequences include the production of RNA transcripts having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring sequence.

The invention also encompasses production of DNA sequences, or portions thereof,

which encode NHCR and its derivatives, entirely by synthetic chemistry. After production, the synthetic sequence may be inserted into any of the many available expression vectors and cell systems using reagents that are well known in the art at the time of the filing of this application. Moreover, synthetic chemistry may be used to introduce mutations into a sequence encoding  
5 NHCR or any portion thereof.

Also encompassed by the invention are polynucleotide sequences that are capable of hybridizing to the claimed nucleotide sequences, and in particular, those shown in SEQ ID NO:2, under various conditions of stringency. Hybridization conditions are based on the melting temperature ( $T_m$ ) of the nucleic acid binding complex or probe, as taught in Wahl, G.M. and S.L.  
10 Berger (1987; Methods Enzymol. 152:399-407) and Kimmel, A.R. (1987; Methods Enzymol. 152:507-511), and may be used at a defined stringency.

Altered nucleic acid sequences encoding NHCR which are encompassed by the invention include deletions, insertions, or substitutions of different nucleotides resulting in a polynucleotide that encodes the same or a functionally equivalent NHCR. The encoded protein may also contain  
15 deletions, insertions, or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent NHCR. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the biological activity of NHCR is retained. For example, negatively charged amino acids may include aspartic acid and glutamic acid; positively  
20 charged amino acids may include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values may include leucine, isoleucine, and valine; glycine and alanine; asparagine and glutamine; serine and threonine; phenylalanine and tyrosine.

Also included within the scope of the present invention are alleles of the genes encoding NHCR. As used herein, an "allele" or "allelic sequence" is an alternative form of the gene which  
25 may result from at least one mutation in the nucleic acid sequence. Alleles may result in altered mRNAs or polypeptides whose structure or function may or may not be altered. Any given gene may have none, one, or many allelic forms. Common mutational changes which give rise to alleles are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times  
30 in a given sequence.

Methods for DNA sequencing which are well known and generally available in the art may be used to practice any embodiments of the invention. The methods may employ such enzymes as the Klenow fragment of DNA polymerase I, Sequenase® (US Biochemical Corp,

Cleveland, OH), Taq polymerase (Perkin Elmer), thermostable T7 polymerase (Amersham, Chicago, IL), or combinations of recombinant polymerases and proofreading exonucleases such as the ELONGASE Amplification System marketed by Gibco BRL (Gaithersburg, MD).

Preferably, the process is automated with machines such as the Hamilton Micro Lab 2200

- 5 (Hamilton, Reno, NV), Peltier Thermal Cycler (PTC200; MJ Research, Watertown, MA) and the ABI 377 DNA sequencers (Perkin Elmer).

The nucleic acid sequences encoding NHCR may be extended utilizing a partial nucleotide sequence and employing various methods known in the art to detect upstream sequences such as promoters and regulatory elements. For example, one method which may be  
10 employed, "restriction-site" PCR, uses universal primers to retrieve unknown sequence adjacent to a known locus (Sarkar, G. (1993) PCR Methods Applic. 2:318-322). In particular, genomic DNA is first amplified in the presence of primer to linker sequence and a primer specific to the known region. The amplified sequences are then subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round  
15 of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

Inverse PCR may also be used to amplify or extend sequences using divergent primers based on a known region (Triglia, T. et al. (1988) Nucleic Acids Res. 16:8186). The primers may be designed using OLIGO 4.06 Primer Analysis software (National Biosciences Inc., Plymouth,  
20 MN), or another appropriate program, to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68°-72° C. The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template.

Another method which may be used is capture PCR which involves PCR amplification of  
25 DNA fragments adjacent to a known sequence in human and yeast artificial chromosome DNA (Lagerstrom, M. et al. (1991) PCR Methods Applic. 1:111-119). In this method, multiple restriction enzyme digestions and ligations may also be used to place an engineered double-stranded sequence into an unknown portion of the DNA molecule before performing PCR.

30 Another method which may be used to retrieve unknown sequences is that of Parker, J.D. et al. (1991; Nucleic Acids Res. 19:3055-3060). Additionally, one may use PCR, nested primers, and PromoterFinder™ libraries to walk in genomic DNA (Clontech, Palo Alto, CA). This process avoids the need to screen libraries and is useful in finding intron/exon junctions.

When screening for full-length cDNAs, it is preferable to use libraries that have been size-selected to include larger cDNAs. Also, random-primed libraries are preferable, in that they will contain more sequences which contain the 5' regions of genes. Use of a randomly primed library may be especially preferable for situations in which an oligo d(T) library does not yield a full-length cDNA. Genomic libraries may be useful for extension of sequence into the 5' and 3' non-transcribed regulatory regions.

Capillary electrophoresis systems which are commercially available may be used to analyze the size or confirm the nucleotide sequence of sequencing or PCR products. In particular, capillary sequencing may employ flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled device camera. Output/light intensity may be converted to electrical signal using appropriate software (e.g. Genotyper™ and Sequence Navigator™, Perkin Elmer) and the entire process from loading of samples to computer analysis and electronic data display may be computer controlled. Capillary electrophoresis is especially preferable for the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample.

In another embodiment of the invention, polynucleotide sequences or fragments thereof which encode NHCR, or fusion proteins or functional equivalents thereof, may be used in recombinant DNA molecules to direct expression of NHCR in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be produced and these sequences may be used to clone and express NHCR.

As will be understood by those of skill in the art, it may be advantageous to produce NHCR-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce a recombinant RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

The nucleotide sequences of the present invention can be engineered using methods generally known in the art in order to alter NHCR encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the gene product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides may be used to engineer the nucleotide sequences. For

example, site-directed mutagenesis may be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, or introduce mutations, and so forth.

In another embodiment of the invention, natural, modified, or recombinant nucleic acid sequences encoding NHCR may be ligated to a heterologous sequence to encode a fusion protein.

5 For example, to screen peptide libraries for inhibitors of NHCR activity, it may be useful to encode a chimeric NHCR protein that can be recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between the NHCR encoding sequence and the heterologous protein sequence, so that NHCR may be cleaved and purified away from the heterologous moiety.

10 In another embodiment, sequences encoding NHCR may be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers, M.H. et al. (1980) Nucl. Acids Res. Symp. Ser. 215-223, Horn, T. et al. (1980) Nucl. Acids Res. Symp. Ser. 225-232). Alternatively, the protein itself may be produced using chemical methods to synthesize the amino acid sequence of NHCR, or a portion thereof. For example, peptide synthesis can be performed  
15 using various solid-phase techniques (Roberge, J.Y. et al. (1995) Science 269:202-204) and automated synthesis may be achieved, for example, using the ABI 431A Peptide Synthesizer (Perkin Elmer).

The newly synthesized peptide may be substantially purified by preparative high performance liquid chromatography (e.g., Creighton, T. (1983) Proteins, Structures and  
20 Molecular Principles, WH Freeman and Co., New York, NY). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; Creighton, supra). Additionally, the amino acid sequence of NHCR, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide.

25 In order to express a biologically active NHCR, the nucleotide sequences encoding NHCR or functional equivalents, may be inserted into appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence.

Methods which are well known to those skilled in the art may be used to construct  
30 expression vectors containing sequences encoding NHCR and appropriate transcriptional and translational control elements. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. Such techniques are described in Sambrook, J. et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press,



Plainview, NY, and Ausubel, F.M. et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY.

A variety of expression vector/host systems may be utilized to contain and express sequences encoding NHCR. These include, but are not limited to, microorganisms such as  
5 bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g., baculovirus); plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids); or animal cell systems.

10 The "control elements" or "regulatory sequences" are those non-translated regions of the vector--enhancers, promoters, 5' and 3' untranslated regions--which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be  
15 used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the Bluescript® phagemid (Stratagene, LaJolla, CA) or pSport1™ plasmid (Gibco BRL) and the like may be used. The baculovirus polyhedrin promoter may be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock, RUBISCO; and storage protein genes) or from plant viruses (e.g., viral promoters or leader  
20 sequences) may be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding NHCR, vectors based on SV40 or EBV may be used with an appropriate selectable marker.

In bacterial systems, a number of expression vectors may be selected depending upon the  
25 use intended for NHCR. For example, when large quantities of NHCR are needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be used. Such vectors include, but are not limited to, the multifunctional E. coli cloning and expression vectors such as Bluescript® (Stratagene), in which the sequence encoding NHCR may be ligated into the vector in frame with sequences for the amino-terminal  
30 Met and the subsequent 7 residues of  $\beta$ -galactosidase so that a hybrid protein is produced; pIN vectors (Van Heeke, G. and S.M. Schuster (1989) J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors (Promega, Madison, WI) may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble

and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems may be designed to include heparin, thrombin, or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

5 In the yeast, Saccharomyces cerevisiae, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH may be used. For reviews, see Ausubel et al. (supra) and Grant et al. (1987) *Methods Enzymol.* 153:516-544.

In cases where plant expression vectors are used, the expression of sequences encoding NHCR may be driven by any of a number of promoters. For example, viral promoters such as the  
10 35S and 19S promoters of CaMV may be used alone or in combination with the omega leader sequence from TMV (Takamatsu, N. (1987) *EMBO J.* 6:307-311). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters may be used (Coruzzi, G. et al. (1984) *EMBO J.* 3:1671-1680; Broglie, R. et al. (1984) *Science* 224:838-843; and Winter, J. et al. (1991) *Results Probl. Cell Differ.* 17:85-105). These constructs can be  
15 introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. Such techniques are described in a number of generally available reviews (see, for example, Hobbs, S. or Murry, L.E. in McGraw Hill Yearbook of Science and Technology (1992) McGraw Hill, New York, NY; pp. 191-196.

An insect system may also be used to express NHCR. For example, in one such system,  
20 Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in Spodoptera frugiperda cells or in Trichoplusia larvae. The sequences encoding NHCR may be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of NHCR will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant  
25 viruses may then be used to infect, for example, S. frugiperda cells or Trichoplusia larvae in which NHCR may be expressed (Engelhard, E.K. et al. (1994) *Proc. Nat. Acad. Sci.* 91:3224-3227).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, sequences encoding NHCR may be  
30 ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing NHCR in infected host cells (Logan, J. and Shenk, T. (1984) *Proc. Natl. Acad. Sci.* 81:3655-3659). In addition, transcription

enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Specific initiation signals may also be used to achieve more efficient translation of sequences encoding NHCR. Such signals include the ATG initiation codon and adjacent  
5 sequences. In cases where sequences encoding NHCR, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading  
10 frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used, such as those described in the literature (Scharf, D. et al. (1994) Results Probl. Cell Differ. 20:125-162).

15 In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding  
20 and/or function. Different host cells such as CHO, HeLa, MDCK, HEK293, and WI38, which have specific cellular machinery and characteristic mechanisms for such post-translational activities, may be chosen to ensure the correct modification and processing of the foreign protein.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express NHCR may be transformed using  
25 expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the  
30 introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M. et al.

(1977) Cell 11:223-32) and adenine phosphoribosyltransferase (Lowy, I. et al. (1980) Cell 22:817-23) genes which can be employed in tk<sup>-</sup> or apt<sup>-</sup> cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, dhfr which confers resistance to methotrexate (Wigler, M. et al. (1980) Proc. Natl. Acad. Sci. 77:3567-70); 5 npt, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin, F. et al (1981) J. Mol. Biol. 150:1-14) and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, supra). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine (Hartman, S.C. and R.C. Mulligan 10 (1988) Proc. Natl. Acad. Sci. 85:8047-51). Recently, the use of visible markers has gained popularity with such markers as anthocyanins,  $\beta$  glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes, C.A. et al. (1995) Methods Mol. Biol. 55:121-131).

15 Although the presence/absence of marker gene expression suggests that the gene of interest is also present, its presence and expression may need to be confirmed. For example, if the sequence encoding NHCR is inserted within a marker gene sequence, recombinant cells containing sequences encoding NHCR can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a sequence encoding NHCR under the 20 control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem gene as well.

Alternatively, host cells which contain the nucleic acid sequence encoding NHCR and express NHCR may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations and 25 protein bioassay or immunoassay techniques which include membrane, solution, or chip based technologies for the detection and/or quantification of nucleic acid or protein.

The presence of polynucleotide sequences encoding NHCR can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes or portions or fragments of polynucleotides encoding NHCR. Nucleic acid amplification based assays involve the use of 30 oligonucleotides or oligomers based on the sequences encoding NHCR to detect transformants containing DNA or RNA encoding NHCR. As used herein "oligonucleotides" or "oligomers" refer to a nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides, preferably about 15 to 30 nucleotides, and more preferably about 20-25 nucleotides,

which can be used as a probe or amplimer.

A variety of protocols for detecting and measuring the expression of NHCR, using either polyclonal or monoclonal antibodies specific for the protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and

5 fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on NHCR is preferred, but a competitive binding assay may be employed. These and other assays are described, among other places, in Hampton, R. et al. (1990; Serological Methods, a Laboratory Manual, APS Press, St Paul, MN) and Maddox, D.E. et al. (1983; J. Exp. Med. 158:1211-1216).

10 A wide variety of labels and conjugation techniques are known by those skilled in the art and may be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding NHCR include oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the sequences encoding NHCR, or any portions thereof may be cloned  
15 into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3, or SP6 and labeled nucleotides. These procedures may be conducted using a variety of commercially available kits (Pharmacia & Upjohn, (Kalamazoo, MI); Promega (Madison WI); and U.S. Biochemical Corp., Cleveland, OH).  
20 Suitable reporter molecules or labels, which may be used, include radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Host cells transformed with nucleotide sequences encoding NHCR may be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The protein  
25 produced by a recombinant cell may be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode NHCR may be designed to contain signal sequences which direct secretion of NHCR through a prokaryotic or eukaryotic cell membrane. Other recombinant constructions may be used to join sequences encoding NHCR to nucleotide  
30 sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the

5 FLAGS extension/affinity purification system (Immunex Corp., Seattle, WA). The inclusion of cleavable linker sequences such as those specific for Factor XA or enterokinase (Invitrogen, San Diego, CA) between the purification domain and NHCR may be used to facilitate purification. One such expression vector provides for expression of a fusion protein containing NHCR and a  
10 nucleic acid encoding 6 histidine residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography as described in Porath, J. et al. (1992, Prot. Exp. Purif. 3: 263-281) while the enterokinase cleavage site provides a means for purifying NHCR from the fusion protein. A discussion of vectors which contain fusion proteins is provided in Kroll, D.J. et al. (1993; DNA  
10 Cell Biol. 12:441-453).

In addition to recombinant production, fragments of NHCR may be produced by direct peptide synthesis using solid-phase techniques Merrifield J. (1963) J. Am. Chem. Soc. 85:2149-2154). Protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide  
15 Synthesizer (Perkin Elmer). Various fragments of NHCR may be chemically synthesized separately and combined using chemical methods to produce the full length molecule.

### THERAPEUTICS

Based on the chemical and structural homology between NHCR (SEQ ID NO:1) and human chemokine receptor R12 (SEQ ID NO:3), NHCR appears to play a role in inflammation  
20 and viral infection.

Therefore, in one embodiment, antagonists or inhibitors of NHCR may be administered to a subject to prevent an inflammatory disorder associated with expression of NHCR. Types of inflammatory disorders may include, but are not limited to, allergic reactions, asthma and adult respiratory distress syndrome, rheumatoid arthritis, osteoarthritis, glomerulonephritis,  
25 osteoporosis, dermatomyositis, polymyositis, Addison's disease, Grave's disease, irritable bowel syndrome, atrophic gastritis, lupus erythematosus, myasthenia gravis, multiple sclerosis, autoimmune thyroiditis, ulcerative colitis, anemia, pancreatitis, scleroderma, Crohn's disease, ischemia/reperfusion injury, post-traumatic inflammation, myocardial inflammation, atherosclerosis, multiple sclerosis, and inflammatory complications of cancer, hemodialysis and  
30 extracorporeal circulation, infection and trauma. In one aspect, antibodies which are specific for NHCR may be used directly as an antagonist, or indirectly as a targeting or delivery mechanism for bringing a pharmaceutical agent to cells or tissue which express NHCR.

In another embodiment, a vector expressing the complement or antisense of the

polynucleotide encoding NHCR may be administered to a subject to treat or prevent the inflammatory disorders associated with NHCR expression listed above.

In another embodiment, antagonists or inhibitors of NHCR may be administered to a subject to prevent a viral infection which is associated with expression of NHCR. Types of viral  
5 infections may include, but are not limited to, HIV, Epstein-Barr virus, VZV, HTLV, CMV, bunyaviruses, influenza, adenovirus, rhinoviruses, hepadnaviruses, and rotaviruses.

In another embodiment, a vector expressing the complement or antisense of the polynucleotide encoding NHCR may be administered to a subject to prevent the viral infections associated with expression of NHCR listed above.

10 In another embodiment, NHCR or a fragment or derivative thereof may be administered to a subject to treat a disorder which is associated with reduced NHCR expression including, but not limited to, complement deficiency, immunodeficiency, sepsis, tumor growth, and impaired wound healing.

In another embodiment, a vector capable of expressing NHCR, or a fragment or a  
15 derivative thereof, may also be administered to a subject to treat a disorder associated with NHCR expression, and particularly those listed in the preceding paragraph.

In other embodiments, any of the therapeutic proteins, antagonists, antibodies, agonists, antisense sequences or vectors described above may be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy  
20 may be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents may act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

25 Antagonists or inhibitors of NHCR may be produced using methods which are generally known in the art. In particular, purified NHCR may be used to produce antibodies or to screen libraries of pharmaceutical agents to identify those which specifically bind NHCR.

The antibodies may be generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain,  
30 Fab fragments, and fragments produced by a Fab expression library. Neutralizing antibodies, (i.e., those which inhibit dimer formation) are especially preferred for therapeutic use.

For the production of antibodies, various hosts including goats, rabbits, rats, mice, humans, and others, may be immunized by injection with NHCR or any fragment or oligopeptide

thereof which has immunogenic properties. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, 5 and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and Corynebacterium parvum are especially preferable.

It is preferred that the peptides, fragments, or oligopeptides used to induce antibodies to NHCR have an amino acid sequence consisting of at least five amino acids, and more preferably at least 10 amino acids. It is also preferable that they are identical to a portion of the amino acid 10 sequence of the natural protein, and they may contain the entire amino acid sequence of a small, naturally occurring molecule. Short stretches of NHCR amino acids may be fused with those of another protein such as keyhole limpet hemocyanin and antibody produced against the chimeric molecule.

Monoclonal antibodies to NHCR may be prepared using any technique which provides for 15 the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (Kohler, G. et al. (1975) Nature 256:495-497; Kozbor, D. et al. (1985) J. Immunol. Methods 81:31-42; Cote, R.J. et al. (1983) Proc. Natl. Acad. Sci. 80:2026-2030; Cole, S.P. et al. (1984) Mol. Cell Biol. 62:109-120).

20 In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison, S.L. et al. (1984) Proc. Natl. Acad. Sci. 81:6851-6855; Neuberger, M.S. et al. (1984) Nature 312:604-608; Takeda, S. et al. (1985) Nature 314:452-454). Alternatively, techniques described for the production of single chain antibodies 25 may be adapted, using methods known in the art, to produce NHCR-specific single chain antibodies. Antibodies with related specificity, but of distinct idiotypic composition, may be generated by chain shuffling from random combinatorial immunoglobulin libraries (Burton D.R. (1991) Proc. Natl. Acad. Sci. 88:11120-3).

Antibodies may also be produced by inducing in vivo production in the lymphocyte 30 population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature (Orlandi, R. et al. (1989) Proc. Natl. Acad. Sci. 86: 3833-3837; Winter, G. et al. (1991) Nature 349:293-299).

Antibody fragments which contain specific binding sites for NHCR may also be



generated. For example, such fragments include, but are not limited to, the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal  
5 Fab fragments with the desired specificity (Huse, W.D. et al. (1989) Science 254:1275-1281).

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between  
10 NHCR and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering NHCR epitopes is preferred, but a competitive binding assay may also be employed (Maddox, supra).

In another embodiment of the invention, the polynucleotides encoding NHCR, or any fragment thereof, or antisense molecules, may be used for therapeutic purposes. In one aspect,  
15 antisense to the polynucleotide encoding NHCR may be used in situations in which it would be desirable to block the transcription of the mRNA. In particular, cells may be transformed with sequences complementary to polynucleotides encoding NHCR. Thus, antisense molecules may be used to modulate NHCR activity, or to achieve regulation of gene function. Such technology is now well known in the art, and sense or antisense oligomers or larger fragments, can be  
20 designed from various locations along the coding or control regions of sequences encoding NHCR.

Expression vectors derived from retro viruses, adenovirus, herpes or vaccinia viruses, or from various bacterial plasmids may be used for delivery of nucleotide sequences to the targeted organ, tissue or cell population. Methods which are well known to those skilled in the art can be  
25 used to construct recombinant vectors which will express antisense molecules complementary to the polynucleotides of the gene encoding NHCR. These techniques are described both in Sambrook et al. (supra) and in Ausubel et al. (supra).

Genes encoding NHCR can be turned off by transforming a cell or tissue with expression vectors which express high levels of a polynucleotide or fragment thereof which encodes NHCR.  
30 Such constructs may be used to introduce untranslatable sense or antisense sequences into a cell. Even in the absence of integration into the DNA, such vectors may continue to transcribe RNA molecules until they are disabled by endogenous nucleases. Transient expression may last for a month or more with a non-replicating vector and even longer if appropriate replication elements

are part of the vector system.

As mentioned above, modifications of gene expression can be obtained by designing antisense molecules, DNA, RNA, or PNA, to the control regions of the gene encoding NHCR, i.e., the promoters, enhancers, and introns. Oligonucleotides derived from the transcription initiation site, e.g., between positions -10 and +10 from the start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. Recent therapeutic advances using triplex DNA have been described in the literature (Gee, J.E. et al. (1994) In: Huber, B.E. and B.I. Carr, Molecular and Immunologic Approaches, Futura Publishing Co., Mt. Kisco, NY). The antisense molecules may also be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Ribozymes, enzymatic RNA molecules, may also be used to catalyze the specific cleavage of RNA. The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples which may be used include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of sequences encoding NHCR.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for secondary structural features which may render the oligonucleotide inoperable. The suitability of candidate targets may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

Antisense molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding NHCR. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7 or SP6. Alternatively, these cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines, cells, or tissues.

RNA molecules may be modified to increase intracellular stability and half-life. Possible

modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the backbone of the molecule. This concept is inherent in the production of PNAs and can be extended in all of these molecules by the inclusion of nontraditional bases such as inosine, queosine, and wybutosine, as well as acetyl-, methyl-, thio-, and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases.

Many methods for introducing vectors into cells or tissues are available and equally suitable for use *in vivo*, *in vitro*, and *ex vivo*. For *ex vivo* therapy, vectors may be introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection and by liposome injections may be achieved using methods which are well known in the art.

Any of the therapeutic methods described above may be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

An additional embodiment of the invention relates to the administration of a pharmaceutical composition, in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed above. Such pharmaceutical compositions may consist of NHCR, antibodies to NHCR, mimetics, agonists, antagonists, or inhibitors of NHCR. The compositions may be administered alone or in combination with at least one other agent, such as stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones.

The pharmaceutical compositions utilized in this invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton,

PA).

Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, 5 pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. 10 Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl 15 pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores may be used in conjunction with suitable coatings, such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for 20 product identification or to characterize the quantity of active compound, i.e., dosage.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft 25 capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations suitable for parenteral administration may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain 30 substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or

triglycerides, or liposomes. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. For administration of NHCR, such labeling would include amount, frequency, and method of administration.

Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, e.g., of neoplastic cells, or in animal models, usually mice, rabbits, dogs, or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

A therapeutically effective dose refers to that amount of active ingredient, for example NHCR or fragments thereof, antibodies of NHCR, agonists, antagonists or inhibitors of NHCR, which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED<sub>50</sub> (the dose therapeutically effective in 50% of the population) and LD<sub>50</sub> (the dose lethal to 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, LD<sub>50</sub>/ED<sub>50</sub>. Pharmaceutical compositions which

exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

## 20 DIAGNOSTICS

In another embodiment, antibodies which specifically bind NHCR may be used for the diagnosis of conditions or diseases characterized by expression of NHCR, or in assays to monitor patients being treated with NHCR, agonists, antagonists or inhibitors. The antibodies useful for diagnostic purposes may be prepared in the same manner as those described above for therapeutics. Diagnostic assays for NHCR include methods which utilize the antibody and a label to detect NHCR in human body fluids or extracts of cells or tissues. The antibodies may be used with or without modification, and may be labeled by joining them, either covalently or non-covalently, with a reporter molecule. A wide variety of reporter molecules which are known in the art may be used, several of which are described above.

A variety of protocols including ELISA, RIA, and FACS for measuring NHCR are known in the art and provide a basis for diagnosing altered or abnormal levels of NHCR expression. Normal or standard values for NHCR expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to NHCR

under conditions suitable for complex formation. The amount of standard complex formation may be quantified by various methods, but preferably by photometric means. Quantities of NHCR expressed in subject, control and disease, samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the  
5 parameters for diagnosing disease.

In another embodiment of the invention, the polynucleotides encoding NHCR may be used for diagnostic purposes. The polynucleotides which may be used include oligonucleotide sequences, antisense RNA and DNA molecules, and PNAs. The polynucleotides may be used to detect and quantitate gene expression in biopsied tissues in which expression of NHCR may be  
10 correlated with disease. The diagnostic assay may be used to distinguish between absence, presence, and excess expression of NHCR, and to monitor regulation of NHCR levels during therapeutic intervention.

In one aspect, hybridization with PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, encoding NHCR or closely related  
15 molecules, may be used to identify nucleic acid sequences which encode NHCR. The specificity of the probe, whether it is made from a highly specific region, e.g., 10 unique nucleotides in the 5' regulatory region, or a less specific region, e.g., especially in the 3' coding region, and the stringency of the hybridization or amplification (maximal, high, intermediate, or low) will determine whether the probe identifies only naturally occurring sequences encoding NHCR,  
20 alleles, or related sequences.

Probes may also be used for the detection of related sequences, and should preferably contain at least 50% of the nucleotides from any of the NHCR encoding sequences. The hybridization probes of the subject invention may be DNA or RNA and derived from the nucleotide sequence of SEQ ID NO:2 or from genomic sequence including promoter, enhancer  
25 elements, and introns of the naturally occurring NHCR.

Means for producing specific hybridization probes for DNAs encoding NHCR include the cloning of nucleic acid sequences encoding NHCR or NHCR derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, commercially available, and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA  
30 polymerases and the appropriate labeled nucleotides. Hybridization probes may be labeled by a variety of reporter groups, for example, radionuclides such as <sup>32</sup>P or <sup>35</sup>S, or enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

Polynucleotide sequences encoding NHCR may be used for the diagnosis of disorders which are associated with expression of NHCR. Examples of such disorders may include, but are not limited to; allergic reactions, asthma and adult respiratory distress syndrome, inflammatory disorders such as rheumatoid arthritis, osteoarthritis, glomerulonephritis, osteoporosis, dermatomyositis, polymyositis, Addison's disease, Grave's disease, irritable bowel syndrome, atrophic gastritis, lupus erythematosus, myasthenia gravis, multiple sclerosis, autoimmune thyroiditis, ulcerative colitis, anemia, pancreatitis, scleroderma, Crohn's disease, ischemia/reperfusion injury, post-traumatic inflammation, myocardial inflammation, atherosclerosis, multiple sclerosis, HIV infection, Epstein-Barr virus, VZV, HTLV, CMV, bunyaviruses, influenza, adenovirus, rhinoviruses, hepadnaviruses, and rotaviruses sepsis, local tumor destruction, tumor regression, tissue repair; and inflammatory complications of cancer, hemodialysis and extracorporeal circulation, infection and trauma. The polynucleotide sequences encoding NHCR may be used in Southern or northern analysis, dot blot, or other membrane-based technologies; in PCR technologies; or in dip stick, pin, ELISA or chip assays utilizing fluids or tissues from patient biopsies to detect altered NHCR expression. Such qualitative or quantitative methods are well known in the art.

In a particular aspect, the nucleotide sequences encoding NHCR may be useful in assays that detect activation or induction of various cancers, particularly those mentioned above. The nucleotide sequences encoding NHCR may be labeled by standard methods, and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantitated and compared with a standard value. If the amount of signal in the biopsied or extracted sample is significantly altered from that of a comparable control sample, the nucleotide sequences have hybridized with nucleotide sequences in the sample, and the presence of altered levels of nucleotide sequences encoding NHCR in the sample indicates the presence of the associated disease. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or in monitoring the treatment of an individual patient.

In order to provide a basis for the diagnosis of disease associated with expression of NHCR, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, which encodes NHCR, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the



values obtained from normal subjects with those from an experiment where a known amount of a substantially purified polynucleotide is used. Standard values obtained from normal samples may be compared with values obtained from samples from patients who are symptomatic for disease. Deviation between standard and subject values is used to establish the presence of disease.

5        Once disease is established and a treatment protocol is initiated, hybridization assays may be repeated on a regular basis to evaluate whether the level of expression in the patient begins to approximate that which is observed in the normal patient. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

10       With respect to cancer, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or  
15 further progression of the cancer.

Additional diagnostic uses for oligonucleotides designed from the sequences encoding NHCR may involve the use of PCR. Such oligomers may be chemically synthesized, generated enzymatically, or produced from a recombinant source. Oligomers will preferably consist of two nucleotide sequences, one with sense orientation (5'→3') and another with antisense (3'←5'),  
20 employed under optimized conditions for identification of a specific gene or condition. The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for detection and/or quantitation of closely related DNA or RNA sequences.

Methods which may also be used to quantitate the expression of NHCR include  
25 radiolabeling or biotinylating nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated (Melby, P.C. et al. (1993) J. Immunol. Methods, 159:235-244; Duplaa, C. et al. (1993) Anal. Biochem. 229-236). The speed of quantitation of multiple samples may be accelerated by running the assay in an ELISA format where the oligomer of interest is presented in various dilutions and a spectrophotometric or  
30 colorimetric response gives rapid quantitation.

In another embodiment of the invention, the nucleic acid sequences which encode NHCR may also be used to generate hybridization probes which are useful for mapping the naturally occurring genomic sequence. The sequences may be mapped to a particular chromosome or to a

specific region of the chromosome using well known techniques. Such techniques include FISH, FACS, or artificial chromosome constructions, such as yeast artificial chromosomes, bacterial artificial chromosomes, bacterial P1 constructions or single chromosome cDNA libraries as reviewed in Price, C.M. (1993) Blood Rev. 7:127-134, and Trask, B.J. (1991) Trends Genet.

5 7:149-154.

FISH (as described in Verma et al. (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York, NY) may be correlated with other physical chromosome mapping techniques and genetic map data. Examples of genetic map data can be found in the 1994 Genome Issue of Science (265:1981f). Correlation between the location of the gene  
10 encoding NHCR on a physical chromosomal map and a specific disease, or predisposition to a specific disease, may help delimit the region of DNA associated with that genetic disease. The nucleotide sequences of the subject invention may be used to detect differences in gene sequences between normal, carrier, or affected individuals.

In situ hybridization of chromosomal preparations and physical mapping techniques such  
15 as linkage analysis using established chromosomal markers may be used for extending genetic maps. Often the placement of a gene on the chromosome of another mammalian species, such as mouse, may reveal associated markers even if the number or arm of a particular human chromosome is not known. New sequences can be assigned to chromosomal arms, or parts thereof, by physical mapping. This provides valuable information to investigators searching for  
20 disease genes using positional cloning or other gene discovery techniques. Once the disease or syndrome has been crudely localized by genetic linkage to a particular genomic region, for example, AT to 11q22-23 (Gatti, R.A. et al. (1988) Nature 336:577-580), any sequences mapping to that area may represent associated or regulatory genes for further investigation. The nucleotide sequence of the subject invention may also be used to detect differences in the chromosomal  
25 location due to translocation, inversion, etc. among normal, carrier, or affected individuals.

In another embodiment of the invention, NHCR, its catalytic or immunogenic fragments or oligopeptides thereof, can be used for screening libraries of compounds in any of a variety of drug screening techniques. The fragment employed in such screening may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The formation of  
30 binding complexes, between NHCR and the agent being tested, may be measured.

Another technique for drug screening which may be used provides for high throughput screening of compounds having suitable binding affinity to the protein of interest as described in published PCT application WO84/03564. In this method, as applied to NHCR large numbers of

different small test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The test compounds are reacted with NHCR, or fragments thereof, and washed. Bound NHCR is then detected by methods well known in the art. Purified NHCR can also be coated directly onto plates for use in the aforementioned drug screening techniques.

- 5 Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

In another embodiment, one may use competitive drug screening assays in which neutralizing antibodies capable of binding NHCR specifically compete with a test compound for binding NHCR. In this manner, the antibodies can be used to detect the presence of any peptide  
10 which shares one or more antigenic determinants with NHCR.

In additional embodiments, the nucleotide sequences which encode NHCR may be used in any molecular biology techniques that have yet to be developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, including, but not limited to, such properties as the triplet genetic code and specific base pair interactions.

- 15 The examples below are provided to illustrate the subject invention and are not included for the purpose of limiting the invention.

## INDUSTRIAL APPLICABILITY

### I MMLR3DT01 cDNA Library Construction

The normal peripheral blood macrophages used for this library were obtained from two  
20 year old, Caucasian males. This library represents a mixture of allogeneically stimulated human macrophage populations obtained from Ficoll/Hypaque purified buffy coats. The cells from the two different donors (not typed for HLA alleles) were incubated at a density of  $1 \times 10^6$ /ml for 72 hours in DME containing 10% human serum.

After incubation, macrophages mostly adhered to the plastic surface of the petri dish,  
25 whereas most other cell types, B and T lymphocytes, remained in solution. The DME was decanted from the wells, and the wells were washed with phosphate buffered saline (PBS). Macrophages were released from the plastic surface by gently scraping the petri dishes in PBS/1 mM EDTA and lysed immediately in buffer containing guanidinium isothiocyanate.

The lysate was extracted twice with a mixture of acid phenol pH 4.0 and centrifuged over  
30 a CsCl cushion using an Beckman SW28 rotor in a L8-70M Ultracentrifuge (Beckman Instruments). The RNA was precipitated using 0.3 M sodium acetate and 2.5 volumes of ethanol, resuspended in water and DNase treated for 15 min at 37°C. It must be noted that some contaminating T and B lymphocytes may have been present.

The RNA was used in the SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (catalogue #18248-013; Gibco BRL, Gaithersburg MD) with the recommended protocol. cDNAs were fractionated on a Sepharose CL4B column (catalog #275105, Pharmacia), and those cDNAs exceeding 400 bp were ligated into pSport I. The plasmid was transformed into 5 chemically competent DH5 $\alpha$  host cells (Gibco BRL).

## II Isolation and Sequencing of cDNA Clones

Plasmid DNA was released from the cells and purified using the Miniprep Kit (Catalogue # 77468; Advanced Genetic Technologies Corporation, Gaithersburg MD). This kit consists of a 96 well block with reagents for 960 purifications. The recommended protocol was employed 10 except for the following changes: 1) the 96 wells were each filled with only 1 ml of sterile Terrific Broth (Catalog # 22711, Gibco/BRL, Gaithersburg MD) with carbenicillin at 25 mg/L and glycerol at 0.4%; 2) the bacteria were cultured for 24 hours after the wells were inoculated and then lysed with 60  $\mu$ l of lysis buffer; 3) a centrifugation step employing the Beckman GS-6R @2900 rpm for 5 min was performed before the contents of the block were added to the primary 15 filter plate; and 4) the optional step of adding isopropanol to TRIS buffer was not routinely performed. After the last step in the protocol, samples were transferred to a Beckman 96-well block for storage.

The cDNAs were sequenced by the method of Sanger F and AR Coulson (1975; J Mol Biol 94:441f), using a Hamilton Micro Lab 2200 (Hamilton, Reno NV) in combination with four 20 Peltier Thermal Cyclers (PTC200 from MJ Research, Watertown MA) and Applied Biosystems 377 or 373 DNA Sequencing Systems (Perkin Elmer) and reading frame was determined.

## III Homology Searching of cDNA Clones and Their Deduced Proteins

Each cDNA was compared to sequences in GenBank using a search algorithm developed by Applied Biosystems and incorporated into the INHERIT™ 670 sequence analysis system. In 25 this algorithm, Pattern Specification Language (TRW Inc, Los Angeles, CA) was used to determine regions of homology. The three parameters that determine how the sequence comparisons run were window size, window offset, and error tolerance. Using a combination of these three parameters, the DNA database was searched for sequences containing regions of homology to the query sequence, and the appropriate sequences were scored with an initial value. 30 Subsequently, these homologous regions were examined using dot matrix homology plots to distinguish regions of homology from chance matches. Smith-Waterman alignments were used to display the results of the homology search.

Peptide and protein sequence homologies were ascertained using the INHERIT- 670

sequence analysis system using the methods similar to those used in DNA sequence homologies. Pattern Specification Language and parameter windows were used to search protein databases for sequences containing regions of homology which were scored with an initial value. Dot-matrix homology plots were examined to distinguish regions of significant homology from chance

5 matches.

BLAST, which stands for Basic Local Alignment Search Tool (Altschul, S.F. (1993) J. Mol. Evol. 36:290-300; Altschul et al. (1990) J. Mol. Biol. 215:403-410), was used to search for local sequence alignments. BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments,

10 BLAST is especially useful in determining exact matches or in identifying homologs. BLAST is useful for matches which do not contain gaps. The fundamental unit of BLAST algorithm output is the High-scoring Segment Pair (HSP).

An HSP consists of two sequence fragments of arbitrary but equal lengths whose alignment is locally maximal and for which the alignment score meets or exceeds a threshold or

15 cutoff score set by the user. The BLAST approach is to look for HSPs between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. The parameter E establishes the statistically significant threshold for reporting database sequence matches. E is interpreted as the upper bound of the expected frequency of chance occurrence of

20 an HSP (or set of HSPs) within the context of the entire database search. Any database sequence whose match satisfies E is reported in the program output.

#### IV Northern Analysis

Northern analysis is a laboratory technique used to detect the presence of a transcript of a gene and involves the hybridization of a labeled nucleotide sequence to a membrane on which

25 RNAs from a particular cell type or tissue have been bound (Sambrook et al., supra).

Analogous computer techniques using BLAST (Altschul, S.F. 1993 and 1990, supra) are used to search for identical or related molecules in nucleotide databases such as GenBank or the LIFESEQ™ database (Incyte Pharmaceuticals). This analysis is much faster than multiple, membrane-based hybridizations. In addition, the sensitivity of the computer search can be

30 modified to determine whether any particular match is categorized as exact or homologous.

The basis of the search is the product score which is defined as:

$$\frac{\% \text{ sequence identity} \times \% \text{ maximum BLAST score}}{100}$$

The product score takes into account both the degree of similarity between two sequences and the length of the sequence match. For example, with a product score of 40, the match will be exact within a 1-2% error; and at 70, the match will be exact. Homologous molecules are usually identified by selecting those which show product scores between 15 and 40, although lower scores may identify related molecules.

The results of northern analysis are reported as a list of libraries in which the transcript encoding NHCR occurs. Abundance and percent abundance are also reported. Abundance directly reflects the number of times a particular transcript is represented in a cDNA library, and percent abundance is abundance divided by the total number of sequences examined in the cDNA library.

#### V Extension of NHCR-Encoding Polynucleotides

Nucleic acid sequence of Incyte clone 568987 or SEQ ID NO:2 is used to design oligonucleotide primers for extending a partial nucleotide sequence to full length or for obtaining 5' or 3', intron or other control sequences from genomic libraries. One primer is synthesized to initiate extension in the antisense direction (XLR) and the other is synthesized to extend sequence in the sense direction (XLF). Primers are used to facilitate the extension of the known sequence "outward" generating amplicons containing new, unknown nucleotide sequence for the region of interest. The initial primers are designed from the cDNA using OLIGO 4.06 (National Biosciences), or another appropriate program, to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68°-72° C. Any stretch of nucleotides which would result in hairpin structures and primer-primer dimerizations is avoided.

The original, selected cDNA libraries, or a human genomic library are used to extend the sequence; the latter is most useful to obtain 5' upstream regions. If more extension is necessary or desired, additional sets of primers are designed to further extend the known region.

By following the instructions for the XL-PCR kit (Perkin Elmer) and thoroughly mixing the enzyme and reaction mix, high fidelity amplification is obtained. Beginning with 40 pmol of each primer and the recommended concentrations of all other components of the kit, PCR is performed using the Peltier Thermal Cycler (PTC200; M.J. Research, Watertown, MA) and the following parameters:

Step 1	94° C for 1 min (initial denaturation)
Step 2	65° C for 1 min
Step 3	68° C for 6 min
Step 4	94° C for 15 sec

- |    |         |  |
|----|---------|--|
|    | Step 5  | 65° C for 1 min                          |
|    | Step 6  | 68° C for 7 min                          |
|    | Step 7  | Repeat step 4-6 for 15 additional cycles |
|    | Step 8  | 94° C for 15 sec                         |
| 5  | Step 9  | 65° C for 1 min                          |
|    | Step 10 | 68° C for 7:15 min                       |
|    | Step 11 | Repeat step 8-10 for 12 cycles           |
|    | Step 12 | 72° C for 8 min                          |
| 10 | Step 13 | 4° C (and holding)                       |

A 5-10  $\mu$ l aliquot of the reaction mixture is analyzed by electrophoresis on a low concentration (about 0.6-0.8%) agarose mini-gel to determine which reactions were successful in extending the sequence. Bands thought to contain the largest products are selected and removed from the gel. Further purification involves using a commercial gel extraction method such as QIAQuick™ (QIAGEN Inc., Chatsworth, CA). After recovery of the DNA, Klenow enzyme is used to trim single-stranded, nucleotide overhangs creating blunt ends which facilitate religation and cloning.

After ethanol precipitation, the products are redissolved in 13  $\mu$ l of ligation buffer, 1  $\mu$ l T4-DNA ligase (15 units) and 1  $\mu$ l T4 polynucleotide kinase are added, and the mixture is incubated at room temperature for 2-3 hours or overnight at 16° C. Competent *E. coli* cells (in 40  $\mu$ l of appropriate media) are transformed with 3  $\mu$ l of ligation mixture and cultured in 80  $\mu$ l of SOC medium (Sambrook et al., supra). After incubation for one hour at 37° C, the whole transformation mixture is plated on Luria Bertani (LB)-agar (Sambrook et al., supra) containing 2x Carb. The following day, several colonies are randomly picked from each plate and cultured in 150  $\mu$ l of liquid LB/2x Carb medium placed in an individual well of an appropriate, commercially-available, sterile 96-well microtiter plate. The following day, 5  $\mu$ l of each overnight culture is transferred into a non-sterile 96-well plate and after dilution 1:10 with water, 5  $\mu$ l of each sample is transferred into a PCR array.

For PCR amplification, 18  $\mu$ l of concentrated PCR reaction mix (3.3x) containing 4 units of rTth DNA polymerase, a vector primer, and one or both of the gene specific primers used for the extension reaction are added to each well. Amplification is performed using the following conditions:

- |    |        |  |
|----|--------|--|
|    | Step 1 | 94° C for 60 sec                             |
|    | Step 2 | 94° C for 20 sec                             |
| 35 | Step 3 | 55° C for 30 sec                             |
|    | Step 4 | 72° C for 90 sec                             |
|    | Step 5 | Repeat steps 2-4 for an additional 29 cycles |
|    | Step 6 | 72° C for 180 sec                            |

**Step 7                      4° C (and holding)**

Aliquots of the PCR reactions are run on agarose gels together with molecular weight markers. The sizes of the PCR products are compared to the original partial cDNAs, and appropriate clones are selected, ligated into plasmid, and sequenced.

**5 VI            Labeling and Use of Hybridization Probes**

Hybridization probes derived from SEQ ID NO:2 are employed to screen cDNAs, genomic DNAs, or mRNAs. Although the labeling of oligonucleotides, consisting of about 20 base-pairs, is specifically described, essentially the same procedure is used with larger cDNA fragments. Oligonucleotides are designed using state-of-the-art software such as OLIGO 4.06  
10 (National Biosciences), labeled by combining 50 pmol of each oligomer and 250  $\mu$ Ci of [ $\gamma$ - $^{32}$ P] adenosine triphosphate (Amersham) and T4 polynucleotide kinase (DuPont NEN®, Boston, MA). The labeled oligonucleotides are substantially purified with Sephadex G-25 superfine resin column (Pharmacia & Upjohn). A portion containing  $10^7$  counts per minute of each of the sense and antisense oligonucleotides is used in a typical membrane based hybridization analysis of  
15 human genomic DNA digested with one of the following endonucleases (Ase I, Bgl II, Eco RI, Pst I, Xba I, or Pvu II; DuPont NEN®).

The DNA from each digest is fractionated on a 0.7 percent agarose gel and transferred to nylon membranes (Nytran Plus, Schleicher & Schuell, Durham, NH). Hybridization is carried out for 16 hours at 40°C. To remove nonspecific signals, blots are sequentially washed at room  
20 temperature under increasingly stringent conditions up to 0.1 x saline sodium citrate and 0.5% sodium dodecyl sulfate. After XOMAT AR™ film (Kodak, Rochester, NY) is exposed to the blots in a Phosphorimager cassette (Molecular Dynamics, Sunnyvale, CA) for several hours, hybridization patterns are compared visually.

**VII    Antisense Molecules**

25            Antisense molecules to the NHCR-encoding sequence, or any part thereof, is used to inhibit in vivo or in vitro expression of naturally occurring NHCR. Although use of antisense oligonucleotides, comprising about 20 base-pairs, is specifically described, essentially the same procedure is used with larger cDNA fragments. An oligonucleotide based on the coding sequences of NHCR, as shown in Figures 1A, 1B, 1C, 1D and 1E, is used to inhibit expression of  
30 naturally occurring NHCR. The complementary oligonucleotide is designed from the most unique 5' sequence as shown in Figures 1A, 1B, 1C, 1D and 1E and used either to inhibit transcription by preventing promoter binding to the upstream nontranslated sequence or translation of an NHCR-encoding transcript by preventing the ribosome from binding. Using an



appropriate portion of the signal and 5' sequence of SEQ ID NO:2, an effective antisense oligonucleotide includes any 15-20 nucleotides spanning the region which translates into the signal or 5' coding sequence of the polypeptide as shown in Figures 1A, 1B, 1C, 1D and 1E.

### VIII Expression of NHCR

5 Expression of NHCR is accomplished by subcloning the cDNAs into appropriate vectors and transforming the vectors into host cells. In this case, the cloning vector, pSport, previously used for the generation of the cDNA library is used to express NHCR in *E. coli*. Upstream of the cloning site, this vector contains a promoter for  $\beta$ -galactosidase, followed by sequence containing the amino-terminal Met, and the subsequent seven residues of  $\beta$ -galactosidase. Immediately  
10 following these eight residues is a bacteriophage promoter useful for transcription and a linker containing a number of unique restriction sites.

Induction of an isolated, transformed bacterial strain with IPTG using standard methods produces a fusion protein which consists of the first eight residues of  $\beta$ -galactosidase, about 5 to 15 residues of linker, and the full length protein. The signal residues direct the secretion of  
15 NHCR into the bacterial growth media which can be used directly in the following assay for activity.

### IX Demonstration of NHCR Activity

GTP-binding activity is assayed by incubating varying amounts of NHCR protein for 10 minutes at 30°C in 50mM Tris buffer, pH 7.5, containing 1mM dithiothreitol, 1mM EDTA, 1 $\mu$ M  
20 ( $\alpha$ -<sup>32</sup>P), in the absence or presence of 100 $\mu$ M of the following compounds: GTP, GDP, GTP $\gamma$ S, ATP, CTP, UTP, and TTP. Samples are passed through nitrocellulose filters and washed twice with a buffer consisting of 50mM Tris-HCL, pH 7.8, 1mM NaN<sub>3</sub>, 10mM MgCl<sub>2</sub>, 1mM EDTA, 0.5mM dithiothreitol, 0.01mM PMSF, and 200mM NaCl. The filter-bound counts are determined by liquid scintillation. To determine GTPase activity, BND protein is incubated for  
25 10 minutes at 37°C in 50mM Tris-HCL buffer, pH 7.8, containing 1mM dithiothreitol, 2mM EDTA, 10 $\mu$ M ( $\alpha$ -<sup>32</sup>P), and 1 $\mu$ M H-rab protein. GTPase activity is initiated by adding MgCl<sub>2</sub> to a final concentration of 10 mM. Samples are removed at various time points, mixed with an equal volume of ice-cold 0.5mM EDTA, and frozen. Aliquots are spotted onto polyethyleneimine-cellulose thin layer chromatography plates, which are developed in 1M LiCl, dried, and  
30 autoradiographed.

### X Production of NHCR Specific Antibodies

NHCR that is substantially purified using PAGE electrophoresis (Sambrook, supra), or other purification techniques, is used to immunize rabbits and to produce antibodies using

standard protocols. The amino acid sequence deduced from SEQ ID NO:2 is analyzed using DNASTAR software (DNASTAR Inc) to determine regions of high immunogenicity and a corresponding oligopeptide is synthesized and used to raise antibodies by means known to those of skill in the art. Selection of appropriate epitopes, such as those near the C-terminus or in hydrophilic regions, is described by Ausubel et al. (supra), and others.

Typically, the oligopeptides are 15 residues in length, synthesized using an Applied Biosystems Peptide Synthesizer Model 431A using fmoc-chemistry, and coupled to keyhole limpet hemocyanin (KLH, Sigma, St. Louis, MO) by reaction with N-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Ausubel et al., supra). Rabbits are immunized with the oligopeptide-KLH complex in complete Freund's adjuvant. The resulting antisera are tested for antipeptide activity, for example, by binding the peptide to plastic, blocking with 1% BSA, reacting with rabbit antisera, washing, and reacting with radioiodinated, goat anti-rabbit IgG.

#### **XI Purification of Naturally Occurring NHCR Using Specific Antibodies**

Naturally occurring or recombinant NHCR is substantially purified by immunoaffinity chromatography using antibodies specific for NHCR. An immunoaffinity column is constructed by covalently coupling NHCR antibody to an activated chromatographic resin, such as CnBr-activated Sepharose (Pharmacia & Upjohn). After the coupling, the resin is blocked and washed according to the manufacturer's instructions.

Media containing NHCR is passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of NHCR (e.g., high ionic strength buffers in the presence of detergent). The column is eluted under conditions that disrupt antibody/NHCR binding (eg, a buffer of pH 2-3 or a high concentration of a chaotrope, such as urea or thiocyanate ion), and NHCR is collected.

#### **XII Identification of Molecules Which Interact with NHCR**

NHCR or biologically active fragments thereof are labeled with <sup>125</sup>I Bolton-Hunter reagent (Bolton et al. (1973) Biochem. J. 133: 529). Candidate molecules previously arrayed in the wells of a multi-well plate are incubated with the labeled NHCR, washed and any wells with labeled NHCR complex are assayed. Data obtained using different concentrations of NHCR are used to calculate values for the number, affinity, and association of NHCR with the candidate molecules.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit

of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are  
5 intended to be within the scope of the following claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT: INCYTE PHARMACEUTICALS, INC.
- (ii) TITLE OF THE INVENTION: NOVEL HUMAN CHEMOKINE RECEPTOR-LIKE PROTEIN
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Incyte Pharmaceuticals, Inc.
  - (B) STREET: 3174 Porter Dr.
  - (C) CITY: Palo Alto
  - (D) STATE: CA
  - (E) COUNTRY: USA
  - (F) ZIP: 94304
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Diskette
  - (B) COMPUTER: IBM Compatible
  - (C) OPERATING SYSTEM: DOS
  - (D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
- (A) PCT APPLICATION NUMBER: To Be Assigned
  - (B) FILING DATE: Filed Herewith
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: US 08/812,871
  - (B) FILING DATE: 06-MAR-1997
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Billings, Lucy J.
  - (B) REGISTRATION NUMBER: 36,749
  - (C) REFERENCE/DOCKET NUMBER: PF-0237 PCT
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 650-855-0555
  - (B) TELEFAX: 650-845-4166

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 333 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: MMLR3DT01
- (B) CLONE: 568987

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Asn Thr Thr Val Met Gln Gly Phe Asn Arg Ser Glu Arg Cys Pro  
1 5 10 15

Arg Asp Thr Arg Ile Val Gln Leu Val Phe Pro Ala Leu Tyr Thr Val  
 20 25 30  
 Val Phe Leu Thr Gly Ile Leu Leu Asn Thr Leu Ala Leu Trp Val Phe  
 35 40 45  
 Val His Ile Pro Ser Ser Ser Thr Phe Ile Ile Tyr Leu Lys Asn Thr  
 50 55 60  
 Leu Val Ala Asp Leu Ile Met Thr Leu Met Leu Pro Phe Lys Ile Leu  
 65 70 75 80  
 Ser Asp Ser His Leu Ala Pro Trp Gln Leu Arg Ala Phe Val Cys Arg  
 85 90 95  
 Phe Ser Ser Val Ile Phe Tyr Glu Thr Met Tyr Val Gly Ile Val Leu  
 100 105 110  
 Leu Gly Leu Ile Ala Phe Asp Arg Phe Leu Lys Ile Ile Arg Pro Leu  
 115 120 125  
 Arg Asn Ile Phe Leu Lys Lys Pro Val Phe Ala Lys Thr Val Ser Ile  
 130 135 140  
 Phe Ile Trp Phe Phe Leu Phe Phe Ile Ser Leu Pro Ile Met Ile Leu  
 145 150 155 160  
 Ser Asn Lys Glu Ala Thr Pro Ser Ser Val Lys Lys Cys Ala Ser Leu  
 165 170 175  
 Lys Gly Pro Leu Gly Leu Lys Trp His Gln Met Val Asn Asn Ile Cys  
 180 185 190  
 Gln Phe Ile Phe Trp Thr Val Leu Ile Leu Met Leu Val Phe Tyr Val  
 195 200 205  
 Val Ile Ala Lys Lys Val Tyr Asp Ser Tyr Arg Lys Ser Lys Cys Lys  
 210 215 220  
 Asp Arg Lys Asn Asn Lys Lys Leu Glu Gly Lys Val Phe Val Val Val  
 225 230 235 240  
 Pro Val Phe Phe Val Cys Phe Ala Pro Phe His Phe Ala Arg Val Pro  
 245 250 255  
 Tyr Thr His Ser Gln Thr Asn Asn Lys Thr Asp Cys Arg Leu Gln Asn  
 260 265 270  
 Gln Leu Phe Ile Ala Lys Glu Thr Thr Leu Phe Leu Ala Ala Thr Asn  
 275 280 285  
 Ile Cys Met Asp Pro Leu Ile Ser Ile Phe Leu Cys Lys Lys Phe Thr  
 290 295 300  
 Glu Lys Leu Pro Cys Met Gln Gly Arg Lys Thr Thr Ala Ser Ser Gln  
 305 310 315 320  
 Glu Asn His Ser Ser Gln Thr Asp Asn Ile Thr Leu Gly  
 325 330

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1488 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (vii) IMMEDIATE SOURCE:

- (A) LIBRARY: MMLR3DT01
- (B) CLONE: 568987

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACTAGTTCAA	GAGGCCATCT	ACGAACGTAT	GACTGCCGCT	TTAAGAAGAC	AGAGAGAACT	60
GAGTATCCTC	CCAAAGGTGA	CACTGGAAGC	AATGAACACC	ACAGTGATGC	AAGGCTTCAA	120
CAGATCTGAG	CGGTGCCCCA	GAGACACTCG	GATAGTACAG	CTGGTATTCC	CAGCCCTCTA	180
CACAGTGGTT	TTCTTGACCG	GCATCCTGCT	GAATACTTTG	GCTCTGTGGG	TGTTTGTTC	240
CATCCCCAGC	TCCTCCACCT	TCATCATCTA	CCTCAAAAAC	ACTTTGGTGG	CCGACTTGAT	300

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AATGACACTC ATGCTTCCTT TCAAAATCCT CTCTGACTCA CACCTGGCAC CCTGGCAGCT 360
CAGAGCTTTT GTGTGTCGTT TTTCTTCGGT GATATTTTAT GAGACCATGT ATGTGGGCAT 420
CGTGCTGTTA GGGCTCATAG CCTTTGACAG ATTCCTCAAG ATCATCAGAC CTTTGAGAAA 480
TATTTTTCTA AAAAAACCTG TTTTTCGAAA AACGGTCTCA ATCTTCATCT GGTTCTTTTT 540
GTTCTTCATC TCCCTGCCAA TTATGATCTT GAGCAACAAG GAAGCAACAC CATCGTCTGT 600
GAAAAAGTGT GCTTCCTTAA AGGGGCTCTT GGGGCTGAAA TGGCATCAAA TGGTAAATAA 660
CATATGCCAG TTTATTTTCT GGACTGTTTT AATCCTAATG CTTGTGTTTT ATGTGGTTAT 720
TGCAAAAAAA GTATATGATT CTTATAGAAA GTCCAAATGT AAGGACAGAA AAAACAACAA 780
AAAGCTGGAA GGCAAAGTAT TTGTTGTCGT GCCTGTCTTC TTTGTGTGTT TTGCTCCATT 840
TCATTTTGCC AGAGTTCAT ATACTCACAG TCAAACCAAC AATAAGACTG ACTGTAGACT 900
GCAAAATCAA CTGTTTATTG CTAAAGAAAC AACTCTCTTT TTGGCAGCAA CTAACATTTG 960
TATGGATCCC TTAATATCCA TATCTTATG TAAAAAATTC ACAGAAAAGC TACCATGTAT 1020
GCAAGGGAGA AAGACCACAG CATCAAGCCA AGAAAATCAT AGCAGTCAGA CAGACAACAT 1080
AACCTTAGGC TGACAACTGT ACATAGGGTT AACTTCTATT TATTGATGAG ACTTCCGTAG 1140
ATAATGTGGA AATCAAATTT AACCAAGAAA AAAAGATTGG AACAAATGCT CTCTTACATT 1200
TTATTATCCT GGTGTACAGA AAAGATTATA TAAAATTTAA ATCCACATAG ATCTATTTCAT 1260
AAGCTGAATG AACCATTACT AAGAGAATGC AACAGGATAC AAATGGCCAC TAGAGGTCAT 1320
TATTTCTTTC TTTCTTTTTT TTTTTTTTTA ATTTCAAGAG CATTTCACTT TAACATTTTG 1380
GAAAAGACTA AGGAGAAACG TATATCCCTA CAAACCTCCC CTCTAAACAC CTTCTCACAT 1440
TTTTTTCAC AATTACATA AACTACTGC TTTGTGCCCC TTAAATGT 1488

```

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 339 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (vii) IMMEDIATE SOURCE:

- (A) LIBRARY: GenBank
- (B) CLONE: 992700

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

Met Asn Gly Leu Glu Val Ala Pro Pro Gly Leu Ile Thr Asn Phe Ser
 1           5           10           15
Leu Ala Thr Ala Glu Gln Cys Gly Gln Glu Thr Pro Leu Glu Asn Met
           20           25           30
Leu Phe Ala Ser Phe Tyr Leu Leu Asp Phe Ile Leu Ala Leu Val Gly
           35           40           45
Asn Thr Leu Ala Leu Trp Leu Phe Ile Arg Asp His Lys Ser Gly Thr
           50           55           60
Pro Ala Asn Val Phe Leu Met His Leu Ala Val Ala Asp Leu Ser Cys
           65           70           75           80
Val Leu Val Leu Pro Thr Arg Leu Val Tyr His Phe Ser Gly Asn His
           85           90           95
Trp Pro Phe Gly Glu Ile Ala Cys Arg Leu Thr Gly Phe Leu Phe Tyr
           100          105          110
Leu Asn Met Tyr Ala Ser Ile Tyr Phe Leu Thr Cys Ile Ser Ala Asp
           115          120          125
Arg Phe Leu Ala Ile Val His Pro Val Lys Ser Leu Lys Leu Arg Arg
           130          135          140
Pro Leu Tyr Ala His Leu Ala Cys Ala Phe Leu Trp Val Val Val Ala
           145          150          155          160
Val Ala Met Ala Pro Leu Leu Val Ser Pro Gln Thr Val Gln Thr Asn
           165          170          175
His Thr Val Val Cys Leu Gln Leu Tyr Arg Glu Lys Ala Ser His His
           180          185          190

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Ala	Leu	Val	Ser	Leu	Ala	Val	Ala	Phe	Thr	Phe	Pro	Phe	Ile	Thr	Thr
	195						200					205			
Val	Thr	Cys	Tyr	Leu	Leu	Ile	Ile	Arg	Ser	Leu	Arg	Gln	Gly	Leu	Arg
	210					215					220				
Val	Glu	Lys	Arg	Leu	Lys	Thr	Lys	Ala	Val	Arg	Met	Ile	Ala	Ile	Val
	225				230					235					240
Leu	Ala	Ile	Phe	Leu	Val	Cys	Phe	Val	Pro	Tyr	His	Val	Asn	Arg	Ser
			245						250					255	
Val	Tyr	Val	Leu	His	Tyr	Arg	Ser	His	Gly	Ala	Ser	Cys	Ala	Thr	Gln
		260						265					270		
Arg	Ile	Leu	Ala	Leu	Ala	Asn	Arg	Ile	Thr	Ser	Cys	Leu	Thr	Ser	Leu
	275					280						285			
Asn	Gly	Ala	Leu	Asp	Pro	Ile	Met	Tyr	Phe	Phe	Val	Ala	Glu	Lys	Phe
	290					295					300				
Arg	His	Ala	Leu	Cys	Asn	Leu	Leu	Cys	Gly	Lys	Arg	Leu	Lys	Gly	Pro
	305				310					315					320
Pro	Pro	Ser	Phe	Glu	Gly	Lys	Thr	Asn	Glu	Ser	Ser	Leu	Ser	Ala	Lys
				325					330					335	
Ser	Glu	Leu													

What is claimed is: -

1. A substantially purified human chemokine receptor-like protein (NHCR) comprising the amino acid sequence of SEQ ID NO:1 or fragments thereof.
2. An isolated and purified polynucleotide sequence encoding the human chemokine  
5 receptor-like protein of claim 1.
3. A polynucleotide sequence which hybridizes under stringent conditions to the polynucleotide sequence of claim 2.
4. A hybridization probe comprising the polynucleotide sequence of claim 2.
5. An isolated and purified polynucleotide sequence comprising SEQ ID NO:2 or  
10 variants thereof.
6. A polynucleotide sequence which is complementary to the polynucleotide sequence of claim 2 or variants thereof.
7. A hybridization probe comprising the polynucleotide sequence of claim 6.
8. An expression vector containing the polynucleotide sequence of claim 2.
- 15 9. A host cell containing the vector of claim 8.
10. A method for producing a polypeptide comprising the amino acid sequence of SEQ ID NO:1 the method comprising the steps of:
  - a) culturing the host cell of claim 9 under conditions suitable for the expression of the polypeptide; and
  - 20 b) recovering the polypeptide from the host cell culture.
11. A pharmaceutical composition comprising a substantially purified human chemokine receptor-like protein having an amino acid sequence of claim 1 in conjunction with a suitable pharmaceutical carrier.
12. A purified antibody which binds specifically to the polypeptide of claim 1.
- 25 13. A purified agonist which modulates the activity of the polypeptide of claim 1.
14. A purified antagonist which inhibits the activity of the polypeptide of claim 1.
15. A method for treating a disorder which is associated with decreased expression of human chemokine receptor-like protein comprising administering to a subject in need of such treatment an effective amount of the pharmaceutical composition of claim 11.
- 30 16. A pharmaceutical composition comprising the antagonist of claim 14 in conjunction with a suitable pharmaceutical carrier.
17. A method for treating an inflammatory disorder comprising administering to a subject in need of such treatment an effective amount of the pharmaceutical composition of claim



16.

18. A method for treating a viral infection which is associated with expression of human chemokine receptor-like protein comprising administering to a subject in need of such treatment an effective amount of the pharmaceutical composition of claim 16.

5 19. A method for detection of a polynucleotide encoding human chemokine receptor-like protein in a biological sample comprising the steps of:

a) hybridizing the polynucleotide of claim 6 to nucleic acid material of a biological sample, thereby forming a hybridization complex; and

b) detecting said hybridization complex, wherein the presence of said  
10 complex correlates with the presence of a polynucleotide encoding human chemokine receptor-like protein in said biological sample.

CTA GTT CAA GAG GCC ATC TAC GAA CGT ATG ACT GCC GCT TTA AGA AGA CAG AGA	9	18	27	36	45	54
GAA CTG AGT ATC CTC CCA AAG GTG ACA CTG GAA GCA ATG AAC ACC ACA GTG ATG	63	72	81	90	99	108
CAA GGC TTC AAC AGA TCT GAG CGG TGC CCC GAC ACT CGG ATA GTA CAG CTG	117	126	135	144	153	162
Q G F N R S E R C P R D T R I V Q L						
GTA TTC CCA GCC CTC TAC ACA GTG ACA GTG GTT TTC TTG ACC GGC ATC CTG AAT ACT	171	180	189	198	207	216
V F P A L Y T V V F L T G I L N T						
TTG GCT CTG TGG GTG TTT GTT CAC ATC ATC CCC AGC TCC TCC ACC TTC ATC ATC TAC	225	234	243	252	261	270
L A L W V F V H I P S S S T F I Y						
CTC AAA AAC ACT TTG GTG GCC GAC TTG ATA ATG ACA CTC ATG CTG TTC AAA	279	288	297	306	315	324
L K N T L V A D L I M T L M L P F K						

FIGURE 1A

333	ATC CTC TCT GAC TCA CAC CTG GCA CCC TGG CAG CTC	360	AGA GCT TTT GTG TGT CGT	378
	I L S D S H L A P W Q L R A F V C R			
342	TTT TCT TCG GTG ATA TTT TAT GAG ACC ATG TAT GTG	414	GGC ATC GTG CTG TTA GGG	432
	F S S V I F Y Y E T M Y V L L G			
387	CTC ATA GCC TTT GAC AGA TTC CTC AAG ATC ATC AGA	468	CCT TTG AGA AAT ATT TTT	486
	L I A F D R R F L K I I R P L R N I F			
441	CTA AAA CCT GTT TTT GCA AAA ACG GTC TCA ATC TTC	522	ATC TGC TTT TTT TTT	540
	L K K AAA CCT GTT TTT GCA AAA ACG GTC TCA ATC TTC			
495	TTC TTC ATC TCC CTG CCA ATT ATG ATC TTG AGC AAC	576	GAA GCA ACA CCA TCG	594
	F I S L P I M I L S N K E A T P S			
549	TCT GTG AAA AAG TGT GCT TCC TTA AAG GGG CCT CTG	630	GGG CTG AAA TGG CAT CAA	648
	S V K K A C A S L K G P L G L K W H Q			

FIGURE 1B

657	ATG GTA AAT AAC ATA TGC TGC CAG TTT ATT TTC TGG ACT GTT TTA ATC CTA ATG CTT	702
	M V N N I C C Q F I I F W T V L I L M L	
666		675
711	GTG TTT TAT GTG GTT ATT GCA AAA AAG AAA AAT GAT TCT TAT AGA AAG TCC AAA	756
	V F Y V V I I A K K K K V D S Y R K S K	
720		729
765	TGT AAG GAC AGA AAA AAC AAA AAG AAA AAT GGC GAA GGC AAA GTA TTT GTT GTC GTG	810
	C K D R K N N N K K K K L E G K V V V	
774		783
819	CCT GTC TTC TTT GTG TGT TGT CCA TTT CAT TTT GGC AGA GTT CCA TAT ACT	864
	P V F F V C C A P F H F F A R V P Y T	
828		837
873	CAC AGT CAA ACC AAC AAT AAG ACT GAC GAC TGT AGA CTG CAA AAT CAA CTG TTT ATT	918
	H S Q T N N K T D C R L Q N Q L F I	
882		891
927	GCT AAA GAA ACA ACT CTC TTT TTG GCA GCA ACT AAC ATT TGT ATG GAT CCC TTA	972
	A K E T T L F L A A T N I C M D P L	
936		945
963		954

FIGURE 1C

1026  
ATA TCC ATA TTC TTA TGT AAA AAA TTC ACA GAA AAG CTA CCA TGT ATG CAA GGG  
I S I F L C K K K F T E K L P C M Q G  
981 990 999 1008 1017 1026

1080  
AGA AAG ACC ACA GCA TCA AGC CAA GAA AAT CAT AGC AGT CAG ACA GAC AAC ATA  
R K T T A S S S Q E N H S S Q T D N I  
1035 1044 1053 1062 1071 1080

1134  
ACC TTA GGC TGA CAA CTG TAC ATA GGG TTA ACT TCT ATT TAT TGA TGA GAC TTC  
T L G 1089 1098 1107 1116 1125 1134

1188  
CGT AGA TAA TGT GGA AAT CAA ATT TAA CCA AGA AAA AAA GAT TGG AAC AAA TGC  
1143 1152 1161 1170 1179 1188

1242  
TCT CTT ACA TTT TAT TAT CCT GGT GTA CAG AAA AGA TTA TAT AAA ATT TAA ATC  
1197 1206 1215 1224 1233 1242

1296  
CAC ATA GAT CTA TTC ATA AGC TGA ATG AAC CAT TAC TAA GAG AAT GCA ACA GGA  
1251 1260 1269 1278 1287 1296

FIGURE 1D

1305 1314 1323 1332 1341 1350  
TAC AAA TGG CCA CTA GAG GTC ATT ATT TCT TTC TTT CTT TTT TTT TTT TAA

1359 1368 1377 1386 1395 1404  
TTT CAA GAG CAT TTC ACT TTA ACA TTT TGG AAA AGA CTA AGG AGA AAC GTA TAT

1413 1422 1431 1440 1449 1458  
CCC TAC AAA CCT CCC CTC TAA ACA CCT TCT CAC ATT TTT TTC CAC AAT TCA CAT

1467 1476 1485  
AAC ACT ACT GCT TTT GTC CCC TTA AAT GT

FIGURE 1E

1	MNT	- - - -	TVMQGFN	- -	RSERCPRD	TRIV	568987
1	MNGL	EVAP	GLIT	NTFSL	ATAEQ	CGQETPLE	-992700
23	QLVFP	ALYT	VVFL	TGILL	NTLAL	WVFI	568987
31	NMLFA	SFYLL	DFIL	ALVG	NTLAL	WLFIR	-992700
53	SSSTFI	-	IYLN	KT	LVAD	LIMLP	568987
61	KSGT	PANV	FLMHL	AVAD	LSCL	VLPT	-992700
82	DHLAP	WQLRA	FVCR	FS	SVIF	YETMY	568987
91	HFSGN	HWPF	GEIA	CR	LTGF	LFYLN	-992700
112	LLGLI	AFDR	FLKI	IR	PLRN	IFLKK	568987
121	FLT	CISAD	RFLA	IVHP	VKS	LKLRR	-992700
142	VSI	FIW	FFIS	LPIM	ILSN	KEATP	568987
151	ACA	FLW	VVA	-V	AMAP	LLVSP	-992700
172	KCAS	LKGP	LGLKW	HQM	VNNI	CQFI	568987
180	VCLQ	LYRE	KASH	-H	ALVSL	AVAF	-992700
						TF--	

FIGURE 2A

202	L	M	L	V	F	Y	V	V	I	A	K	K	V	Y	D	S	Y	R	K	S	K	C	K	D	R	K	N	N	K	K	568987
207	T	T	V	T	C	Y	L	L	I	I	R	S	L	R	Q	G	L	R	V	-	-	-	-	E	K	R	L	K	T	K	-992700
232	L	E	G	K	V	F	V	V	V	P	V	F	F	V	C	F	A	P	F	H	F	A	R	V	P	Y	T	H	S	Q	568987
233	A	V	R	M	I	A	I	V	L	A	I	F	L	V	C	F	V	P	Y	H	V	N	R	S	V	Y	V	L	H	Y	-992700
262	T	N	N	K	T	D	C	R	L	Q	N	Q	L	F	I	A	K	E	T	T	L	F	L	A	A	T	N	I	C	M	568987
263	R	S	H	G	A	S	C	A	T	Q	R	I	L	A	L	A	N	R	I	T	S	C	L	T	S	L	N	G	A	L	-992700
292	D	P	L	I	S	I	F	L	C	K	K	F	T	E	-	-	-	K	L	P	C	-	-	-	M	Q	G	R	K	T	568987
293	D	P	I	M	Y	F	F	V	A	E	K	F	F	H	A	L	C	N	L	L	C	G	K	R	L	K	G	P	P	P	-992700
316	T	A	S	S	Q	E	N	H	S	S	Q	T	D	N	I	T	L	G													568987
323	S	F	E	G	K	T	N	E	S	S	L	S	A	K	S	E	L														-992700

FIGURE 2B



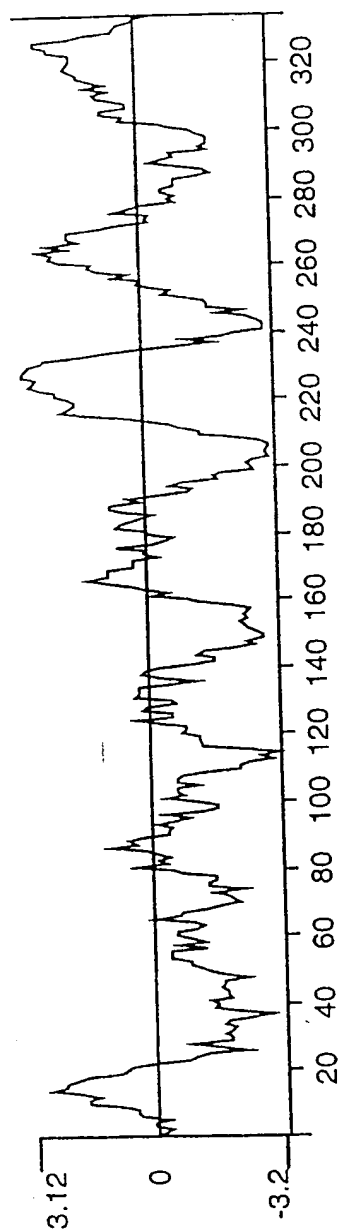


FIGURE 3A

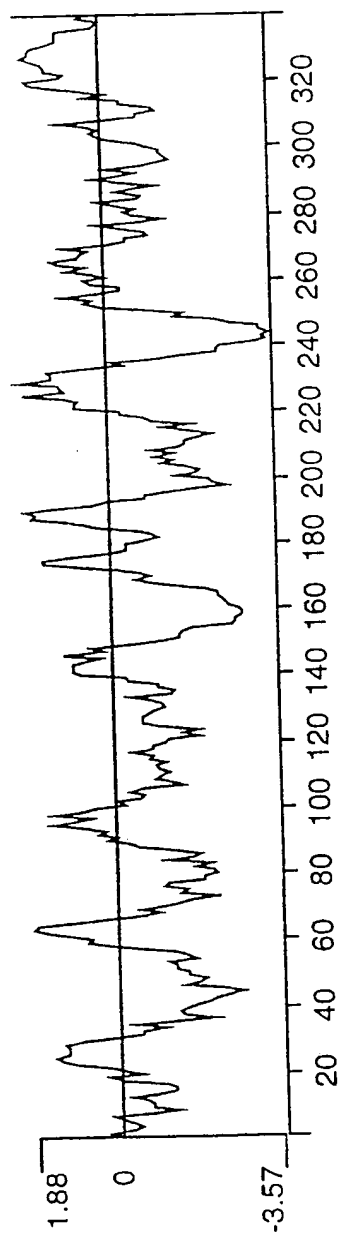


FIGURE 3B

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/04278

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/12 C07K14/715 C07K16/28 A61K38/19 C12Q1/68  
//C12N15/11, C12N9/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N C07K A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 30406 A (HUMAN GENOME SCIENCES INC ; LI YI (US); CAO LIANG (GB); NI JIAN (US) 3 October 1996	1-16, 19
Y	see the whole document, particularly where reference is made to GPR3.	17, 18
Y	WO 96 23068 A (GLAXO GROUP LTD ; WELLS TIMOTHY NIGEL CARL (CH); POWER CHRISTINE AN) 1 August 1996 see the whole document	17, 18

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*G\* document member of the same patent family

Date of the actual completion of the international search

11 June 1998

Date of mailing of the international search report

06.07.98

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Authorized officer

Smalt, R

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 98/04278

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 15, 17 and 18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/04278

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9630406 A	03-10-1996	AU 2236895 A EP 0817800 A	16-10-1996 14-01-1998
WO 9623068 A	01-08-1996	AU 4455896 A EP 0805859 A	14-08-1996 12-11-1997